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Lipase-catalyzed preparation of optically active 1′-acetoxychavicol acetates and their structure–activity relationships in apoptotic activity against human leukemia HL-60 cells

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Abstract—Structure–activity relationships of 1′-acetoxychavicol acetate (**ACA**) for apoptotic activity against human leukemia HL-60 cells were investigated using optically active **ACA** and various racemic **ACA** analogues. Natural-type (or with different acyl group) **ACA** showed a high apoptotic activity, but the *ortho* or *meta* isomers, 4-deacetoxy analogue, and the 2′–3′ dehydrogenated derivative had no effect, or a weak activity. Optically active (*R*)- and (*S*)-**ACA** were prepared by a lipase-catalyzed esterification. Using a mixture of vinyl acetate–tetrahydrofuran (1:1 v/v) as a solvent at refluxing temperature, optically pure (*R*)- and (*S*)-**ACA** were obtained (99.7% ee and 99.1% ee, respectively). The apoptosis-inducing effects of both enantiomers were compared by means of an MTT assay and the detection of typical apoptotic phenomena (DNA fragmentation, caspase-3 activation, and PARP cleavage) and these two activities were almost equal. These results indicate that the essential moieties of **ACA** for apoptotic activity against HL-60 cells are both the presence of a 4-acetoxyl group and an unsaturated double bond between C-2′ and C-3′, and that the configuration at the 1′-position is unrelated to activity.

1. Introduction

1′-Acetoxychavicol acetate (**ACA**), isolated from the rhizomes and the seeds of the Zingiberaceae plant such as *Languas galanga* and *Alpinia galanga*, which is used as a ginger substitute and a stomach medicine in Southeast Asia, has a variety of biological activities (antitumor,[1–3] antiallergic,[4] and repellent[5] effects). **ACA** exerts its antitumor activity by inducing apoptosis in various cancer cells such as Ehrlich ascites,[1] rat and human hepatocellular carcinoma cells,[2] human colon cancer cells,[3] and human myeloid leukemic cells.[6] Morikawa et al.[7] recently reported an inhibitory effect of nitric oxide in mouse peritoneal macrophages by **ACA** isolated from the rhizomes of the Zingiberaceae plant. **ACA** contains a chiral center at the 1′-position and Mitsui et al.[8] determined the stereochemistry at the 1′-position of natural **ACA** as *S*.

The biological effects of **ACA** have been widely investigated, however, in most cases, a natural product or synthetic racemic compound was used in the study. It is likely that the biological activities of the antipode are different from those of the natural form. In fact, Lee and Ando reported on a method for resolving racemic **ACA** by chiral HPLC and demonstrated different repellencies between (*R*)- and (*S*)-**ACA** against female adzuki bean weevils.[5] More recently, Matsuda and colleagues also used optically active **ACA**s resolved by a chiral HPLC and reported that the (*S*)-configuration of the 1′-acetoxyl group was preferable in terms of inhibiting nitric oxide production in lipopolysaccharide-activated

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mouse peritoneal macrophages.[9] The use of chiral HPLC is a convenient method for isolating optically pure compounds on a small scale, but is impractical for use on a large scale.

Lipase is a useful catalyst in organic synthesis for the enantioselective resolution of the racemic compounds in both aqueous solution and organic solvents. Nozaki et al.[10] reported on the efficient lipase-catalyzed (from *Pseudomonas cepacia* sp., lipase PS) preparation of optically active **ACA**s. The enantiomeric excess value of unreacted (*S*)-substrate by their method was slightly low (89.4% ee). Therefore, they repeated the lipase-catalyzed esterification by recycling the (*S*)-substrate after the separation of the acyl product and unreacted substrate, and the final value approached 96.4% ee. However, the authors did not describe the experimental details for this preparation. Thus, we attempted to improve upon their methods so as to produce enantiomerically pure **ACA**s in a single enzymatic procedure.

Previous studies in our laboratories have shown the apoptotic activities of various types of optically active *N*-acetylsphingosine analogues against human leukemia HL-60 cells.[11–14] In the work reported herein, we evaluated the difference in apoptotic activity against HL-60 cells between the natural-type (*S*)-**ACA** and its antipode.

2. Results and discussion

2.1. Syntheses of racemic ACA analogues

Ito and co-workers reported that synthetic, racemic **ACA** induced apoptosis in HL-60 cells.[6] Therefore, we first synthesized various racemic **ACA** analogues to investigate structure–activity relationships in **ACA**-mediated apoptosis (Fig. 1). The racemic **ACA** (*rac*-**ACA**) and its two regio-isomers, *rac*-*o*- and *rac*-*m*-**ACA**, were prepared according to the method reported by Lee and Ando.[5] The diisobutanoyl derivative *rac*-**BCB** was also synthesized using an analogous procedure. The 4-deacetoxy analogue *rac*-**ADC** was prepared as described by Matsuda et al.[9] The saturated derivative *rac*-**DHACA** was obtained as described by Mitsui et al.[8] The 3′-acetoxy analogue, *trans*-*p*-coumaryl diacetate **3′-ACA** which is structurally more stable than the 1′-isomer and in fact, is produced by *A. galanga*,[7] was obtained after rearrangement of the 1′-acetoxy group of *rac*-**ACA** by treatment with AcONa/AcOH.

2.2. Structure–activity relationships of racemic ACA analogues for apoptotic activity against HL-60 cells

The cell death activity of these racemic **ACA** analogues against human leukemia HL-60 cells was evaluated by means of an MTT assay. HL-60 cells were treated with various concentrations (1–20 μM) of each analogue for 6 h. Figure 2A shows that, only naturally occurring **ACA** and its diisobutanoyl derivative induced cell death at a concentration of 10 μM, and that none of the compounds had any effect at a concentration of 1 μM. In contrast to natural-type **ACA**, its two positional

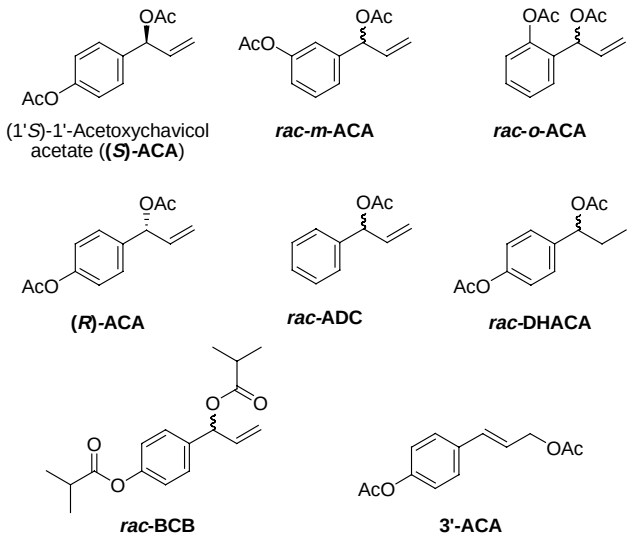


Figure 1. Structures of (1′*S*)-1′-acetoxychavicol acetate ((*S*)-**ACA**) and its analogues.

isomers, *o*- and *m*-**ACA**, and in addition, the 4-deacetoxy analogue *rac*-**ADC** and the saturated derivative *rac*-**DHACA** showed a weak activity. The 3′-acetoxy isomer, **3′-ACA**, also had no effect at a concentration of 10 μM. Figure 2B shows the time course for cell viability (%) when cells were treated with 10 μM of each analogue. The cell death activity of *rac*-**ACA** and *rac*-**BCB** became apparent even at 2 h after their addition, and the percentages of survival cells gradually decreased. *rac*-**ACA** was more effective than its diisobutanoyl derivative *rac*-**BCB** and about 80% of the cells were dead after 4 h. Figure 3 shows the electrophoretic analysis of DNA extracted from cells after treatment with each of the analogues at a concentration of 5 μM for 6 h. Large amounts of fragmented DNA, one of the typical apoptotic phenomena, were observed in the *rac*-**ACA**- and *rac*-**BCB**-treated cells. These results reveal that the essential moieties for apoptotic activity against HL-60 cells are the presence of both a 4-acetoxyl group (or other acyl groups) and an unsaturated double bond between C-2′ and C-3′.

2.3. Preparation of optically pure ACAs using lipase-catalyzed enantioselective esterification

As natural-type, racemic **ACA** had potent apoptotic activity, we attempted to synthesize optically active (*R*)- and (*S*)-**ACA** by enzymatic optical resolution employing Nozaki's method,[8] to investigate the differences in biological effects between these two enantiomers.

The synthetic approach to two chiral **ACA**s is outlined in Scheme 1. Treatment of *p*-hydroxybenzaldehyde (**1**) with *tert*-butyldimethylsilyl chloride in the presence of imidazole followed by a Grignard reaction with vinylmagnesium bromide gave the 4-*O*-protected alcohol **3** in 65% yield from **1**. Using this racemic alcohol as the substrate, a lipase-catalyzed, enantioselective esterification was carried out using various conditions. (*S*)-**ACA** was obtained after the deprotection of the TBS group

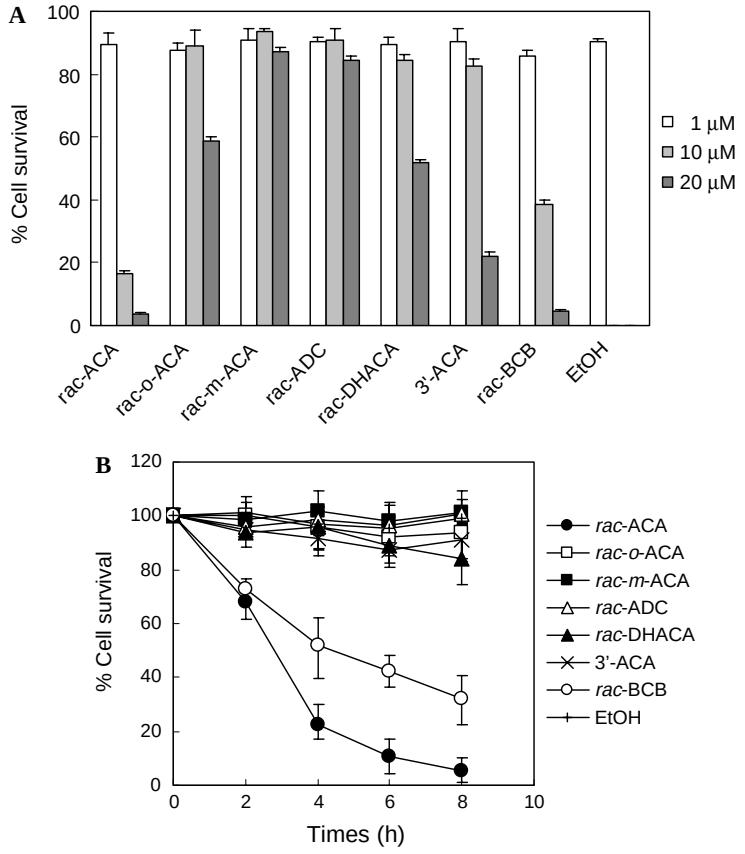


Figure 2. Antileukemic effects of the various racemic **ACA** analogues against HL-60 cells. (A) Cells were treated with the indicated concentrations of each **ACA** for 6 h. (B) Cells were exposed with each ceramide (10 μM) for 2–8 h. Each values are the average of at least three separate experiments.

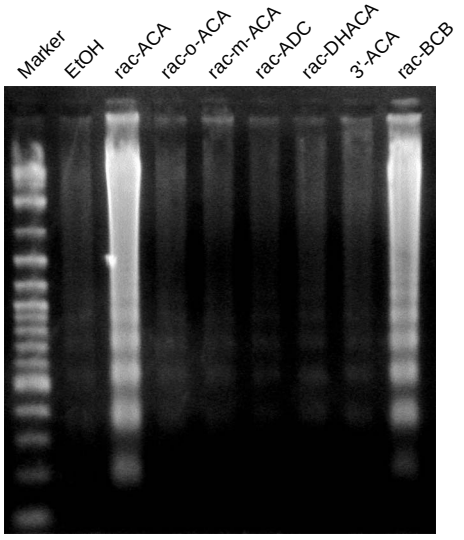
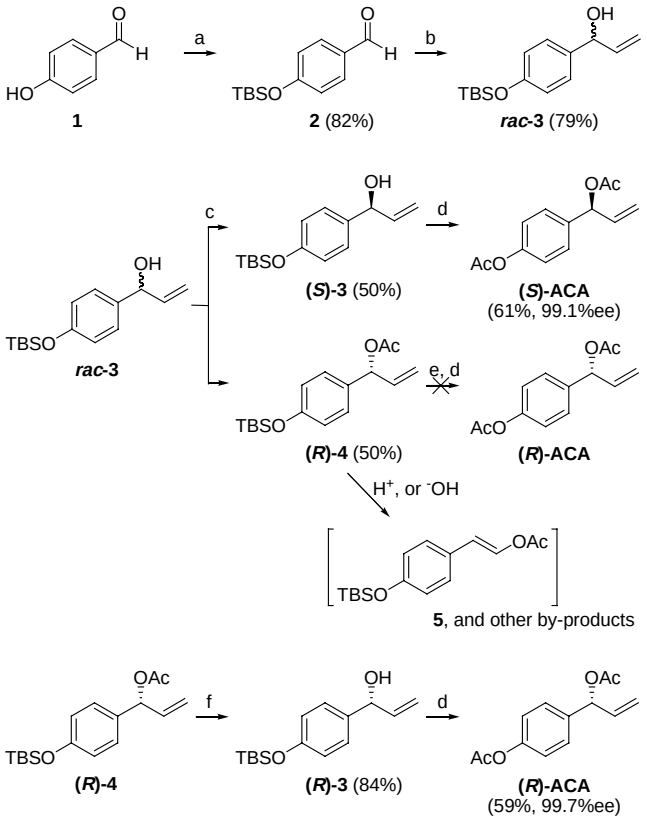


Figure 3. Agarose gel electrophoresis of DNA following treatment of HL-60 cells with racemic **ACA** analogues. HL-60 cells (1×10^6 cells/mL) were exposed to each analogue (5 μM) or ethanol vehicle. After 6 h, genomic DNA was subjected to agarose gel electrophoresis.

of the unreacted (*S*)-**3** by treatment with tetra-*n*-butylammonium fluoride (TBAF) followed by acetylation. Unfortunately, the acetylated product (*R*)-**4** was unstable, as the 1′-acetoxy group of (*R*)-**4** easily rearranges during the purification process using silica gel column chromatography to give the structurally stable 3′-isomer **5**. This rearrangement also occurred during the deprotection of the TBS group using TBAF or under acidic conditions. Thus, it was not possible to isolate the desirable (*R*)-**ACA** after acetylation. Therefore, the deacetylation of (*R*)-**4** was attempted before deprotection of the 4-TBS group. However, even under very weakly alkaline conditions, rearrangement of the 1′-acetoxy group occurred. Therefore, we carried out the lipase-catalyzed hydrolysis according to Nozaki's report.[8] In phosphate buffer (pH 7), the enzymatic hydrolysis of (*R*)-**4** proceeded smoothly without the undesired rearrangement. The conversion values for the enzymatic reaction were estimated by the ^{1}H NMR spectral data. The enantiomeric excess value was obtained from a chiral HPLC analysis of the final product. The results for the optical resolution of racemic **3** are summarized in Table 1.

At first, the effects of additional organic solvents on the catalytic activity and enantioselectivity of lipase were investigated. Mine and co-workers reported that isopropyl ether (IPE) was an optimal organic solvent for both enzyme activity and its enantioselectivity.[15] Our results were also in agreement with their report and the enantioselectivity of lipase in the IPE–vinyl acetate mixture (94.6% ee of (*S*)-**ACA**, lane 2) was slightly higher than in vinyl acetate, only (92.5% ee, lane 1). However, the most efficient solvent was THF under our reaction conditions and both the degree of conversion (50.1%) and



Scheme 1. Synthetic plan for the preparation of (*R*)- and (*S*)-**ACA**. (a) TBSCl, imidazole, DMF, rt; (b) vinylmagnesium bromide, THF, 0 °C; (c) lipase PS, BHT, vinyl acetate–THF (1:1), reflux; (d) TBAF, THF, 0 °C, then Ac_2O, pyridine, rt; (e) K_2CO_3, MeOH–H_2O, rt; (f) lipase PS, 0.1 M phosphate buffer (pH 7.0), rt.

Table 1. Enantioselective acetylation of racemic **3** with vinyl acetate catalyzed by lipase PS

Lane	Solvent	Temp. (°C)	C[a] (%)	Enantiomer excess[b] (%)	
				(*R*)-**ACA**	(*S*)-**ACA**
1	None	65	49.0	98.1	92.5
2	IPE	65	49.9	98.2	94.6
3	THF	65	50.1	99.7	99.1
4	THF	25	48.2	98.0	90.9
5[c]	THF	65	46.4	99.4	84.7
6[c,d]	THF	65	49.1	99.6	97.2

Reaction conditions: substrate (*rac*-**3**, 1 g); lipase PS (1.8 g); vinyl acetate (15 mL); solvent (15 mL); 2,6-di-*tert*-butyl *p*-cresol (25 mg); reaction time: 24 h.

[a] C, conversion (estimated from ^{1}H NMR analysis).

[b] Determined from HPLC analysis.

[c] Half the amount of lipase was used.

[d] Reaction time: 30 h.

the enantiomeric excess of the recovered (*S*)-**ACA** (99.1% ee) were high (lane 3). At room temperature, the enzyme activity was decreased slightly (lane 4). When half the amount of lipase was used under this condition, the enantiomeric excess of (*S*)-**ACA** was greatly reduced (84.7% ee), which was probably caused by the decrease of the degree of conversion (46.4%). However, the enantioselectivity could probably be retained because the enantiomeric excess of (*S*)-**ACA** derived from the acetyl product was 99.4% ee, although this compound was treated with lipase again to hydrolyze the acetyl group after esterification (lane 5). In fact, when the same reaction was carried out for 30 h, the degree of conversion and the enantiomeric excess of (*S*)-**ACA** were improved (49.1% and 97.2% ee, respectively, lane 6).

The $[\alpha]_D^{25}$ values of (*R*)-**ACA** (99.7% ee) and (*S*)-**ACA** (99.1% ee) were +60.0 (*c* 1.282, EtOH) and −60.1 (*c* 1.442, EtOH), respectively. These values are slightly higher than the values reported by Matsuda et al. {(*R*)-**ACA**: $[\alpha]_D^{21}$ +57.1 (*c* 0.28, EtOH); (*S*)-**ACA**: $[\alpha]_D^{22}$ +56.5 (*c* 0.28, EtOH)}.[9]

2.4. Induction of apoptosis by (*R*)- and (*S*)-ACA in HL-60 cells

We investigated the cell death activity of optically active (*R*)- and (*S*)-**ACA** by means of an MTT assay, as described above, using the racemic analogues. Figure 4 shows the time course for cell viability (%) when cells were treated with 5 or 10 μM of these compounds. Both enantiomers exhibited similar antileukemic effects. This suggests that the activities of these two enantiomers are almost equal.

We further investigated the differences in the apoptotic activities between (*R*)- and (*S*)-**ACA** by the detection of typical apoptotic phenomena, DNA fragmentation, caspase-3 activation, and subsequent PARP cleavage. Figure 5 shows the electrophoretic analysis of DNA extracted from cells treated with 5 μM (*R*)- and (*S*)-**ACA** for 6 h. Both enantiomers showed typical DNA fragmentation patterns in HL-60 cells after treatment for 6 h with each **ACA**, as evidenced by the electrophoretic analysis. There was no difference in the degree of fragmented DNA between (*R*)- and (*S*)-**ACA**, consistent with the results of the MTT assay.

Other apoptotic phenomena, caspase-3 activation, which is a well-known executor of apoptosis, and the cleavage of poly(ADP-ribose) polymerase (PARP), a target of proteolytic cleavage by active caspase-3, were detected by Western blot analysis. Caspase-3 is synthesized as a 32 kDa precursor that, after cleavage, results in a 17 kDa subunit (the active form).[16] Active caspase-3 cleaves PARP and generates an 85 kDa fragment.[17] Figure 6A shows the time course for the generated active form of caspase-3 produced by treatment with (*R*)- and (*S*)-**ACA**. Furthermore, PARP was cleaved by treatment with both enantiomers after a delay in caspase activation (Fig. 6B). These results indicate that both (*R*)- and (*S*)-**ACA**-induced apoptosis occurs via the same pathway and that the activities of these two compounds are almost equal.

In conclusion, the findings of this study are summarized as follows. (1) A useful, lipase-catalyzed method for the preparation of optically active (1′*S*)-1′-acetoxychavicol acetate and its antipode was established and both the optical purities of the two isolated enantiomers were high (over 99% ee). (2) The structural requirements of **ACA** for apoptotic activity against HL-60 cells

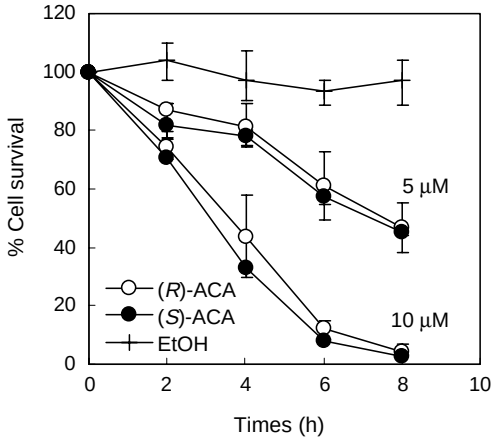


Figure 4. Antileukemic effects of (*R*)- and (*S*)-**ACA** against HL-60 cells. Cells were exposed with each two enantiomers (5 or 10 μM) for 2–8 h. Each value is the average of at least six separate experiments.

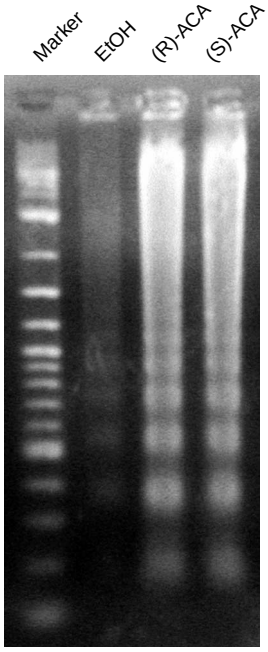


Figure 5. Agarose gel electrophoresis of DNA following treatment of HL-60 cells with (*R*)- and (*S*)-**ACA**. HL-60 cells (1×10^6 cells/mL) were exposed to each enantiomer (5 μM) or ethanol vehicle. After 6 h, genomic DNA was subjected to agarose gel electrophoresis.

were both the presence of a 4-acetoxyl group and an unsaturated double bond between C-2′ and C-3′. This finding is very similar to the structure–activity relationships of natural (*S*)-**ACA** and its related compounds on the release of β-hexosaminidase in rat basophilic leukemia RBL-2H3 cells, as described in Matsuda et al.,[4] although the mechanism is unknown. (3) The 1′-position of the allylic acetoxy group was also important in apoptosis-inducing activity; however, the configuration of the 1′-position of **ACA** was not necessary for activity.

Recently, Ito et al.[19] reported that racemic **ACA** has an inhibitory effect on NF-κB and induces the apoptosis of myeloma cells in vitro and in vivo. Ichikawa and co-

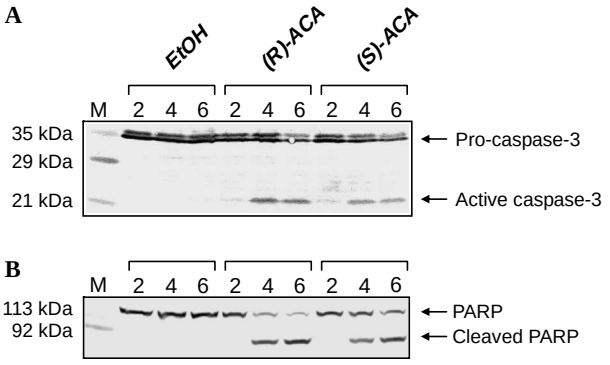


Figure 6. Time-dependent Western blot analysis of (A) caspase-3 and (B) PARP in HL-60 cells with optically active **ACA**s. Cell extracts after treatment with each enantiomer (5 μM) were resolved by SDS-PAGE and probed with *anti*-caspase-3 or *anti*-PARP antibody; M, Marker.

workers suggested that racemic **ACA** regulates NF-κB activity through the inhibition of I-κBα kinase activity.[20] In future, we intend to investigate whether our obtained structure–activity relationships of **ACA** analogues, including its chirality, correlate with the inhibition effect of NF-κB on **ACA**-induced apoptosis.

3. Materials and methods

3.1. Materials

CH_2Cl_2 was distilled from CaH_2 under nitrogen before use. Dehydrated THF and DMF were purchased from WAKO. Lipase PS from *P. cepacia* was obtained from Amano. *n*-Hexane and 2-propanol of HPLC grade were used without further purification. Other materials were obtained commercially (guaranteed reagent grade). Column chromatography was performed on silica gel. The human leukemic HL-60 cells were provided by the Riken Cell Bank (Tsukuba, Japan).

3.2. Chemicals

Racemic *p*-**ACA** (*rac*-**ACA**) and its two positional isomers, *rac*-*o*-**ACA** and *rac*-*m*-**ACA**, were synthesized as described by Lee and Ando.[5] The isobutanoyl analogue, *rac*-**BCB**, was also obtained using isobutyryl chloride instead of Ac_2O.

3.2.1. *rac*-BCB. A colorless oil, IR (NaCl) 2976, 2878, 1759, 1732, 1607, 1508, 1470, 1387, 1348, 1207, 1150, 1018, 939, 868, 815, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 1.17 (d, 3H, $J = 7.1$ Hz), 1.20 (d, 3H, $J = 7.1$ Hz), 1.31 (d, 3H, $J = 7.1$ Hz), 2.61 (septet, 1H, $J = 7.1$ Hz), 2.80 (septet, 1H, $J = 7.1$ Hz), 5.24 (d, 1H, $J = 10.5$ Hz), 5.30 (d, 1H, $J = 17.1$ Hz), 5.98 (ddd, 1H, $J = 5.9$, 10.5, 17.1 Hz), 6.26 (d, 1H, $J = 5.9$ Hz), 7.06 (d, 2H, $J = 8.5$ Hz), 7.36 (d, 2H, $J = 8.5$ Hz); HRMS (EI) calcd for $C_{17}H_{22}O_4$, $[M]^+$ 290.1518; found, 290.1501 (12.5%).

The 4-deacetoxy analogue, *rac*-**ADC**, was prepared as described by Matsuda et al.[9] The saturated derivative *rac*-**DHACA** was synthesized as described by Mitsui et al.[8]

3.2.2. *trans*-*p*-Coumaryl diacetate (3′-ACA). The racemic **ACA** (200 mg, 0.854 mmol) and sodium acetate (200 mg, 2.44 mmol) were dissolved in acetic acid (10 mL). After stirring overnight at 60 °C, the mixture was neutralized with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The organic layer was dried with Na_2SO_4 and concentrated. The obtained residue was purified by column chromatography with *n*-hexane–EtOAc (4:1) to give **3′-ACA** (120 mg, 60%) as a waxy solid; mp 45 °C; IR (KBr) 1759, 1736, 1601, 1508, 1377, 1366, 1254, 1194, 1169, 1103, 1032, 974, 916, 854, 804 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 2.10 (s, 3 Hz), 2.30 (s, 3H), 4.72 (dd, 2H, J = 1.2, 6.3 Hz), 6.24 (dt, 1H, J = 15.9, 6.3 Hz), 6.63 (d, 1H, J = 15.9 Hz), 7.05 (d, 2H, J = 8.8 Hz), 7.39 (d, 2H, J = 8.8 Hz); HRMS (EI) calcd for $C_{13}H_{14}O_4$, $[M]^+$ 234.0892; found, 234.0895 (31.2%).

3.2.3. 4-*tert*-Butyldimethylsiloxybenzaldehyde (2). To a solution of *p*-hydroxybenzaldehyde **1** (2.5 g, 20 mmol) and imidazole (1.96 g, 1.4 equiv) in dry DMF (50 mL), a solution of TBSCl (3.7 g, 1.2 equiv) in dry DMF (10 mL) was added dropwise at 0 °C, and the mixture was stirred for 3 h at room temperature. The resulting mixture was concentrated in vacuo and then diluted with EtOAc. The organic layer was washed with saturated aqueous $NaHCO_3$, dried with Na_2SO_4, and concentrated. The obtained residue was purified by column chromatography with *n*-hexane–EtOAc (20:1) to give **2** (3.87 mg, 82%) as a colorless oil; IR (NaCl) 1703, 1599, 1576, 1506, 1471, 1421, 1393, 1364, 1273, 1211, 1155, 1101, 1007, 907, 841, 800, 783, 717 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.25 (s, 6H), 1.00 (s, 9H), 6.95 (d, 2H, J = 8.5 Hz), 7.79 (d, 2H, J = 6.9 Hz), 9.89 (s, 1H); HRMS (EI) calcd for $C_{13}H_{20}O_2Si$, $[M]^+$ 236.1233; found, 236.1218 (34.4%).

3.2.4. Racemic 4-*O*-*tert*-butyldimethylsilyl-1′-hydroxychavicol (*rac*-3). To a solution of **2** (2 g, 8.48 mmol) in dry THF (20 mL), vinylmagnesium bromide (1 M in THF, 10 mL) was added dropwise at 0 °C. After stirring for 3 h at room temperature, 0.5 M hydrochloric acid (20 mL) was added and then extracted with EtOAc. The organic layer was dried with Na_2SO_4 and concentrated. The obtained residue was purified by column chromatography with *n*-hexane–EtOAc (5:1) to give *rac*-**3** (1.76 g, 79%) as a pale yellow oil; IR (NaCl) 3380, 2957, 2859, 1609, 1508, 1472, 1362, 1256, 1169, 1099, 989, 916, 839, 802, 781, 719 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.19 (s, 6H), 0.98 (s, 9H), 1.89–1.92 (br s, 1H), 5.11–5.17 (m, 1H), 5.18 (d, 1H, J = 10.2 Hz), 5.33 (d, 1H, J = 17.1 Hz), 6.05 (ddd, 1H, J = 5.9, 10.2, 17.1 Hz), 6.82 (d, 1H, J = 8.6 Hz), 7.23 (d, 1H, J = 8.6 Hz); HRMS (EI, direct) calcd for $C_{15}H_{24}O_2Si$, $[M]^+$ 264.1546; found, 264.1545 (25%).

3.2.5. Lipase-catalyzed esterification of *rac*-3. General procedure was carried out according to Sugai's method.[18] To a solution of *rac*-**3** (1 g, 3.78 mmol) and 2,6-di-*tert*-butyl *p*-cresol (a polymerization inhibitor for vinyl acetate, 25 mg, 0.11 mmol) in a mixture of freshly distilled vinyl acetate (15 mL) and dry THF (15 mL), lipase PS (1.8 g) was suspended and stirred for 24 h at 65 °C in dark. After cooling, lipase was removed by filtration and the filtrate was concentrated. The obtained residue was purified by column chromatography with *n*-hexane–EtOAc (10:1 to 5:1) to give crude product (*R*)-**4** (580 mg, slightly including by-product **5**) and unreacted (*S*)-**3** (500 mg, 50%); ^1H NMR (400 MHz, $CDCl_3$); δ 0.19 (s, 6H), 0.98 (s, 9H), 2.09 (s, 3H), 5.22 (d, 1H, J = 10.5 Hz), 5.26 (d, 1H, J = 16.8 Hz), 6.00 (ddd, 1H, J = 5.9, 10.5, 16.8 Hz), 6.22 (d, 1H, J = 5.9 Hz), 6.81 (d, 2H, J = 8.5 Hz), 7.21 (d, 2H, J = 8.5 Hz). Since the 1′-acetoxy group of (*R*)-**4** could be easily rearranged, this compound was used immediately in the next lipase-catalyzed hydrolysis.

3.2.6. Lipase-catalyzed hydrolysis of (*R*)-4. Lipase PS (140 mg) and crude (*R*)-**4** (580 mg) were suspended in 0.1 M phosphate buffer (50 mL, pH 7.0) and stirred for 20 h at room temperature. The reaction mixture was diluted with saturated brine and extracted with EtOAc. The organic layer was dried with Na_2SO_4 and concentrated. The obtained residue was purified by column chromatography with *n*-hexane–EtOAc (5:1) to give (*S*)-**3** (420 mg, 84% from *rac*-**3**).

3.2.7. (1′*S*)-Acetoxychavicol acetate ((*S*)-ACA). To a solution of (*S*)-**3** (500 mg, 1.89 mmol) in dry THF (20 mL), tetra-*n*-butylammonium fluoride (1 M in THF, 2.8 mL) was added dropwise at 0 °C. After stirring for 1 h at 0 °C, the reaction mixture was diluted with saturated brine and extracted with ether, then EtOAc. The combined organic layer was dried with Na_2SO_4 and concentrated. The residue was dissolved in pyridine (20 mL) and acetic anhydride (770 mg, 4 equiv) was added to the solution. After stirring overnight at room temperature, the solvent was removed in vacuo, and 1 M hydrochloric acid was added to the residue, and the mixture was extracted with $CHCl_3$. The organic layer was dried with Na_2SO_4 and concentrated. The obtained residue was purified by column chromatography with *n*-hexane–EtOAc (4:1) to give natural-type (*S*)-**ACA** (270 mg, 61%) as a colorless oil; $[\alpha]_D^{25} = -60.1$ (*c* 1.442, EtOH) {lit.[9] $[\alpha]_D^{22} = -56.5$ (*c* 1, EtOH)}, $[\alpha]_D^{25} = -57.4$ (*c* 1.67, $CHCl_3$); IR (NaCl) 1732, 1645, 1607, 1506, 1371, 1204, 1167, 1096, 1018, 912, 858, 964, 719 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 2.10 (s, 3H), 2.29 (s, 3H), 5.25 (d, 1H, J = 10.5 Hz), 5.30 (d, 1H, J = 17.1 Hz), 5.98 (ddd, 1H, J = 5.9, 10.5, 17.1 Hz), 6.26 (d, 1H, J = 5.9 Hz), 7.08 (d, 2H, J = 8.5 Hz), 7.37 (d, 2H, J = 8.5 Hz); HRMS (FAB, direct) calcd for $C_{13}H_{14}O_4$, $[M]^+$ 234.0892; found, 234.0900 (12%); Anal. Calcd: C, 66.66; H, 6.02. Found: C, 66.54; H, 6.03.

The enantiomer (*R*)-**ACA** was also prepared using compound (*R*)-**3**; $[\alpha]_D^{25} = +60.0$ (*c* 1.282, EtOH) {lit.[9] $[\alpha]_D^{22} = +57.1$ (*c* 0.28, EtOH)}, $[\alpha]_D^{25} = +57.4$ (*c* 1.67, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{13}H_{14}O_4$, $[M]^+$ 234.0892; found, 234.0901 (22%); Anal. Calcd: C, 66.66; H, 6.02. Found: C, 66.37; H, 6.03.

3.3. Analysis

The degree of conversion in the lipase-catalyzed esterification was estimated from the ratio between the

1′-proton signal at δ 6.22 for the acetyl product (*R*)-**4** and the 2′-proton signal at δ 5.95–6.10 for both the product and the unreacted substrate, by the ^{1}H NMR analysis. Enantiomeric excess (ee) values for the prepared **ACA** were determined by the chiral HPLC analysis with a Daicel chiralcel OJ column. Elution was carried out with *n*-hexane–2-propanol (99.7:0.3, v/v) at a flow late of 1 mL/min, and the eluate was monitored at 258 nm: t_S = 71.5 min, t_R = 83.2 min. The evaluation of biological activities was carried out as described in our previous report.[12]

3.4. Cell culture

Human leukemia HL-60 cells were grown in RPMI 1640 medium containing 10% heat-incubated fetal bovine serum. On the day of the experiment, cells were washed twice in serum-free RPMI 1640 and resuspended in the serum-free medium ($0.4–1 \times 10^6$ cells/mL). All **ACA** analogues were dissolved in EtOH. Control experiments were performed with EtOH (0.1%) as the vehicle.

3.5. MTT assay

The cell viability was assessed by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT). MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water-insoluble formazan salt. 4×10^5 cells/100 μL were plated in 96-well dishes. Each **ACA** analogue was dissolved in EtOH at a stock concentration (1–20 mM) and then diluted with serum-free medium. The final concentration of EtOH was 0.1%. Ten microliters of 5 mg/mL MTT was added to each well 2 h before the end of the culture, and the reaction was stopped by adding 100 μL of 0.04 M HCl in 2-propanol. The absorbance at a wavelength of 570 nm was measured (reference: 630 nm). All results were determined as the average of at least three separate experiments.

3.6. DNA fragmentation

HL-60 cells (2×10^6 cells/2 mL) were plated in 6-well dishes. (*R*)- and (*S*)-**ACA** were dissolved in ethanol at a stock concentration of 5 mM and then diluted with serum-free medium. After 6 h incubation, the cells were collected by centrifugation at 1500 rpm for 10 min at 4 °C. Cells were lysed in 100 μL lysis buffer (10 mM Tris–HCl; pH 7.4, 10 mM EDTA; pH 8.0, and 0.5% Triton X-100). Soluble cell lysates were collected by centrifugation at 15,000 rpm for 5 min. Cell lysates were treated for 1 h at 37 °C with RNase A (0.2 mg/mL). Proteinase K (0.2 mg/mL) was added, and the sample was incubated at 50 °C for 30 min. 5 M NaCl (20 μL) and isopropanol (120 μL) were added and the sample was incubated at −20 °C for one night. DNA pellets were collected by centrifugation at 15,000 rpm for 15 min and dissolved in 20 μL of a TE buffer (10 mM Tris–HCl; pH 7.4, 1 mM EDTA; pH 8.0). The DNA was then electrophoresed at 50 V through 2.0% agarose gel. The DNA bands were visualized under UV light after staining with ethidium bromide.

3.7. Immunoblot analysis

2×10^6 cells/2 mL were plated in 6-well dishes. Each **ACA** analogue was dissolved in EtOH at a stock concentration of 5 mM and then diluted with serum-free medium. The final concentration of EtOH was 0.1%. After several hours of incubation, cells were collected by centrifugation at 1500 rpm for 10 min, washed twice with phosphate buffer, pH 7.4, and lysed in 100 mL lysis buffer (62.5 mM Tris–HCl; pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, and 5% β-mercaptoethanol). Cell lysates were boiled for 3 min and separated on 14% (for caspase-3) or 8% (for PARP) SDS–polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and probed with rabbit polyclonal anti-caspase-3 antibody (Santa Cruz Biotechnology) or monoclonal anti-PARP antibody (Trevigen), followed by goat anti-rabbit antibody (for Caspase-3) or anti-mouse antibody (for PARP) coupled to alkaline phosphatase (Sigma).

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